Identification and characterization of potential inducers of aldehyde dehydrogenase from food phytochemicals

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PREFACE

The experiments described in the dissertation were carried out at the Graduate School of Environmental and Life Science (Doctor course), Okayama University, Japan, from October, 2014 to September 2017, under the supervision of Professor Y. Nakamura. These studies are original work by the author and any other assistance and collaboration from others are specially acknowledged.

This dissertation has not been submitted previously whole or in the part to the council, a university or any other professional institution for a degree, diploma or other professional qualification.

YUJIA LIU

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ABBREVIATIONS

ALDH, aldehyde dehydrogenase;

AhR, aryl hydrocarbon receptor;

AA, acetaldehyde;

ARE, antioxidant response element;

BITC, Benzyl isothiocyanate;

DOPAC, 3,4-Dihydroxyphenylacetic acid;

HO-1, heme oxygenase-1;

GST, glutathione S-transferases;

GCLC, glutamate-cysteine ligase catalytic subunit;

NQO1, NAD(P)H: quinone oxidoreductase 1;

Nrf2, nuclear factor erythroid 2-related factor 2;

NF-κB, Nuclear factor κB;

NAD^+, β-nicotinamide-adenine dinucleotide, oxidized form;

Q4′G, quercetin 4′-glucoside;

ABSTRACT

Ethanol elimination is an important detoxified metabolism after ethanol absorption, which mainly contains two steps. The first step is catalyzed by alcohol dehydrogenase, which converted ethanol to acetaldehyde. In the second step, aldehyde dehydrogenase (ALDH) catalyzes the toxic aldehydes to the nontoxic acids. In Asian countries, about 40% of the people have the mutation of ALDH2, which is called ALDH2*2. This mutation leads to the accumulation of acetaldehyde which causes significant damage to the liver and serious abnormal reaction of the body like vasodilation and facial flushing. Food phytochemicals, such as polyphenols and organosulfur compounds, have been confirmed to have various promising effects on health promotion. In this study, I have been focusing on the induction of the ALDH activity by food phytochemicals to prevent the acetaldehyde-induced cytotoxicity.

In the Chapter 1, I determined the effect of benzyl isothiocyanate (BITC) on the ALDH activity in mouse hepatoma Hepa1c1c7 cells. BITC, an ingredient in papaya, is a member of isothiocyanates (ITCs) mainly derived from the cruciferous vegetables. ITCs have been reported to show several beneficial biological effects, including enhanced detoxification against carcinogens and reactive oxygen species. BITC was shown to enhance not only the total ALDH activity, but also the ALDH activity of the cytosol/microsomal and mitochondrial fractions. BITC also increased the total ALDH activity in rat normal liver RL34 cells and human hepatocellular carcinoma HepaG2 cells. BITC significantly increased the gene expression of ALDH1A1, ALDH2 and ALDH3A1 in a concentration-dependent manner. BITC also increased the protein expression of ALDH1A1, ALDH2 and ALDH3A1, suggesting that BITC enhanced the total ALDH

enzyme activity through a transcriptional regulation. Simultaneously, the gene expression of phase 2 drug-metabolizing enzymes, such as NAD(P)H: quinone oxidoreductase 1 and heme oxygenase-1, was increased by the BITC treatment. Western blot experiments revealed that BITC not only up-regulated the Nrf2 protein expression, but also stimulated the nuclear translocation of Nrf2. Furthermore, silencing Nrf2 reduced the basal and BITC-enhanced levels of the total ALDH activity and gene expression of ALDHs. The pretreatment of BITC completely mitigated the acetaldehyde-induced cytotoxicity, which was impaired by silencing Nrf2. The present study demonstrated that BITC is identified as a potential inducer of the total ALDH activity to prevent the acetaldehyde-induced cytotoxicity.

In the Chapter 2, I examined the effects of 3,4-dihydroxyphenylacetic acid (DOPAC), a major colonic microflora-produced catabolite of quercetin glycosides, on the total ALDH activity in Hepa1c1c7 cells. DOPAC was shown to significantly increase the total ALDH activity. DOPAC also significantly increased each gene expression of ALDH1A1, ALDH2 and ALDH3A1. Even though DOPAC did not change the total protein expression of Nrf2, AhR, and NF-κB too much, the nuclear levels of Nrf2 and AhR, but not NF-κB, significantly increased after the DOPAC treatment. These results suggested that DOPAC is able to activate the signaling pathways of both Nrf2 and AhR, but rather inhibit the NF-κB pathway. Furthermore, the pretreatment of 10 μM DOPAC for 6 h completely impaired the acetaldehyde-induced cytotoxicity. These results suggested that DOPAC impaired the acetaldehyde-induced cytotoxicity, possibly through the activation of the AhR and Nrf2 pathways with the inhibition of the NF-κB pathway.

The present study provides biological evidences that (1) an organosulfur compound, BITC, and a phenolic acid, DOPAC, are potential enhancers of the total ALDH activity in Hepa1c1c7 cells; (2) BITC enhanced the total ALDH activity, possibly through a

transcriptional factor Nrf2; (3) Pretreatment of BITC attenuated the acetaldehyde-induced cytotoxicity, also through the Nrf2 pathway; (4) DOPAC impaired acetaldehyde-induced cytotoxicity, possibly through the activation of the AhR and Nrf2 pathways; Taken together, the present study represents a potentially efficient strategy to prevent the alcohol-induced abnormal reaction.

CHAPTER 1

General Introduction

1.1 Ethanol metabolism

Alcohol, i.e., ethanol, is a small molecule soluble in both water and lipids. It is absorbed into blood from the stomach to the small intestine, then distributed throughout the body. The liver is the most functional organ for ethanol detoxification. Ethanol is first metabolized to corresponding aldehyde by alcohol dehydrogenase (ADH) and then aldehyde is metabolized by aldehyde dehydrogenase (ALDH) to acid (Matysiak-Budnik et al., 1996). Acetaldehyde, the most toxic metabolite of ethanol, it also can be directly absorbed from food and tobacco smoke (Yan et al., 2016). Accumulation of acetaldehyde can cause a serious of diseases, such as mitochondrial dysfunction, liver damage and gut barrier dysfunction (Setshedi et al., 2010). Mitochondrial ALDH2 is a member of ALDH family, specially catalyzes acetaldehyde to acetate. ALDH2 ameliorates alcohol-induced liver injury through oxidation of acetaldehyde (Tanaka et al., 2016). ALDH1A1 and ALDH3A1 are also belong to ALDH family which have been reported assistant effect of ALDH2 in acetaldehyde oxidation (Chen et al., 2015)(Lind et al., 2008). In addition, ALDH1A1 play an important role in the formation of retinoic acid which is a potent modulator for gene expression and tissue differentiation (Vasiliou and Nebert, 2005). ALDH3A1, a high proportion of mammalian cornea, protect against UV-induced oxidative stress through cellular lipids peroxidation (Reisdorph and Lindahl, 2007).

1.1.2 ALDH2 mutant

ALDH2*2 allele is ALDH2 mutant encodes a protein subunit with a lysine for glutamate substitution at position 487 which causes flush reaction (Xiao et al., 1995). ALDH2*2 protein expressed as an increased Km for NAD^+ and reduced V max. This

change in Km for NAD^+ renders the enzyme nearly inactive at the concentration of NAD^+ that occurs in cells and the clearance of acetaldehyde is decreased (Xiao et al., 1996). ALDH2*2 allele also increased sensitivity to acute or chronic alcohol-induced toxicity. When exposed to a chemical carcinogen, ALDH2*2 allele pronounced liver injury and accelerated developed to hepatocellular carcinoma (HCC)(Abe et al., 2015)(Jin et al., 2015).

1.2 Food phytochemicals

Food phytochemicals are compounds found in plant such as vegetables, fruits, nuts and other edible plants. Many famous food phytochemicals such as polyphenols, flavanols, glucosinolates have been reported their protective effect on cancer and other chronic diseases (Scalbert et al., 2011).

1.2.1 Benzyl isothiocyanate

Benzyl isothiocyanate (BITC) is one of isothiocyanates (ITCs) which are derived from cruciferous vegetables, also is an extremely rich source in papaya seeds (Nakamura et al., 2007). Epidemiologic studies suggest that consumption of cruciferous vegetables is protective against cancers (Higdon et al., 2007). A wide variety of ITCs have been reported to show several beneficial biological effects including enhanced detoxification against carcinogens and reactive oxygen species. We previously demonstrated that benzyl ITC (BITC) induces phase 2 drug-metabolizing enzymes such as GSTs (Nakamura et al., 2000). BITC inhibits cell proliferation by inducing cell cycle arrest or apoptosis in hepatocytes (Nakamura et al., 2002), T lymphocytes (Miyoshi et al., 2004), colon fibroblasts (Miyoshi et al., 2007), cervical epithelial cells (Miyoshi et al., 2008), renal proximal tubular cells (Abe et al., 2012), and colorectal cancer cells (Abe et al., 2014). In addition, BITC reduces inflammatory responses in macrophages (Murakami

et al., 2003) and the allergy-related cytokine expression in basophil cells (Tang et al., 2015).

1.2.2 Quercetin

Quercetin is one of the flavono naturally occurring in onions, apples, tea and wine as the glycosides (Hubbard et al., 2006). It has been shown to exert anticancer and anti-inflammatory effects (Srivastava et al., 2016). Quercetin 4'-glucoside (Q4'G) is one of the quercetin conjugates in onion, which is absorbed more efficiently than another typical monoglucoside quercetin 3'-glucoside (Q3'G)(Day et al., 2003). Many studies have shown that Q4'G is a good resource of mononglucoside which can prevent oxidative damage (Hollman et al., 1997)(Graefe et al., 2001). Mullen et al. used radiolabeled Q4'G ([2-14C] Q4'G) to monitor the metabolites. After 6 h ingestion, the flavonol glucoside was converted to glucuronide and methylated and sulfated derivatives of quercetin in the small intestine. Continually the flavonol metabolites declined and finally converted to phenolic acids, principally 3-hydroxyphenylacetic acid (OPAC) and 3,4-dihydroxyphenylacetic acid (DOPAC), by the colonic microflora on entering the cecum and the colon (Mullen et al., 2008).

1.3 Transcriptional factors involved in ALDH activity

1.3.1 Keap1/Nrf2/ARE pathway

The Keap1/Nrf2/ARE pathway is an important regulator of cytoprotective responses to endogenous and exogenous reactive oxygen species (ROS) stress and electrophiles (Baird and Dinkova-Kostova, 2011). Transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) translocated from cytoplasm to nucleus where it binds together with small Maf proteins to the antioxidant response element (ARE) in the regulatory regions of target genes (Kensler et al., 2007). ALDH1a1, ALDH2 and ALDH3a1 is probably

mediated by the Keap1/Nrf2/ARE pathway because ARE core consensus sequences were identified in the 5-flanking region of their genes (Sreerama and Sládek, 2001)(Azmannabdullah et al., 2012)(Ushida and Talalay, 2013).

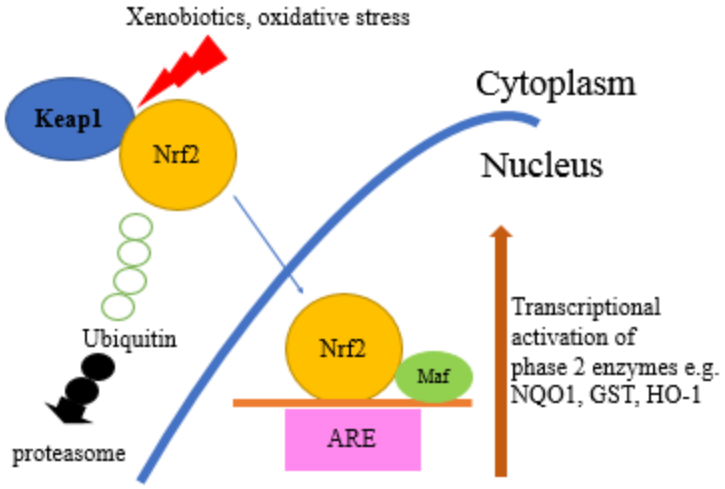


Fig. 1.1 Diagram of the activation of Nrf2

1.3.2 AhR/ARNT/XRE pathway

The AhR is a cytosolic receptor which the most widely recognized ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and regulate gene expression of Cyp1a1, NQO1 and ALDH3A1 (Kurita et al., 2014). On ligand binding, AhR binds to the aryl hydrocarbon receptor nuclear transporter (ARNT) and translocates to the nucleus (Brauze et al., 2006). The AhR-ARNT heterodimer promotes the transcription of genes containing xenobiotic response elements (XRE) in their promoters (Boesch et al., 1999). The traditional role of AhR is metabolizing environmental toxins, moreover recently AhR have been shown its effect on the development, cellular oxidation/antioxidation, responses to ultraviolet light, melanogenesis, epidermal barrier function, and immune

regulation (Esser and Rannug, 2015). A previous study demonstrated that the knockdown of AhR reduced the total ALDH activity by 80% in Hs578T cells (Stanford et al., 2016). AhR also interacted with RAR/RXR receptors which has modified effect on ALDH1 (Bunaciu and Yen, 2011).

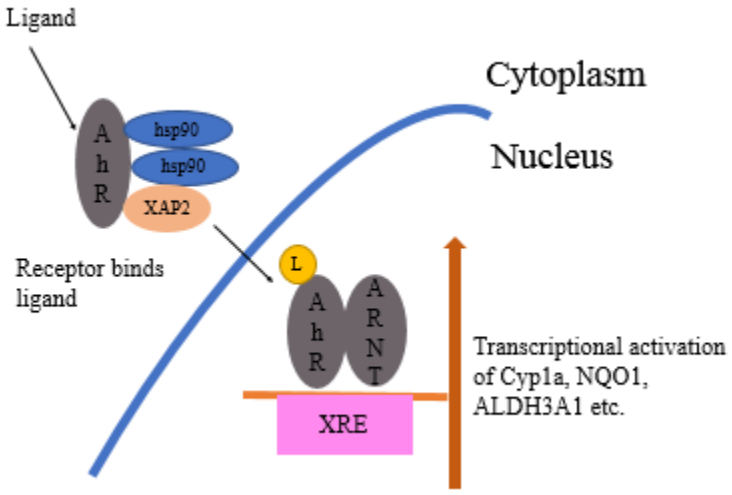


Fig. 1.2 Diagram of the activation of AhR

1.3.3 Nuclear factor-kappa B

The transcription factor NF-κB is a cytosolic protein complex which control DNA transcription, it translocated to nucleus when activated. NF-κB is found in almost all animal cell types and famous for regulating the immune response to infection (Oeckinghaus and Ghosh, 2009). R.A. Canuto et al. reported that Docosahexaenoic acid inhibited ALDH3A1 expression by decrease of AP-1 and NF-κB binding activity (Muzio et al., 2006).

1.4 Study outlines

In the present study, I assessed two food phytochemicals BITC and DOPAC as potential ALDH enhancers to prevent against acetaldehyde-induced cytotoxicity used Hepa1c1c7 cells. BITC also increase ALDH activity in RL 34 and HepG2 cells. I also investigated the mechanism involved in BITC or DOPAC induced ALDH activity in Hepa1c1c7 cells.

My findings provide biological evidence that (1) BITC not only enhanced ALDH activity, but also increased gene expression of ALDH1A1, ALDH2 and ALDH3A1 which provided a possibility that BITC enhanced ALDH activity at transcriptional level; (2) BITC increased gene expression of Nrf2 regulatory genes NQO1 and HO-1. And BITC increased Nrf2 protein expression and stimulated Nrf2 nuclear translocation; (3) Silencing Nrf2 affected the total ALDH activity and each gene expression of ALDHs; (4) BITC pretreatment increased the resistance to the acetaldehyde-induced cytotoxicity through Nrf2 pathway; (5) DOPAC is an enhancer of ALDH activity and ALDH expression; (6) Transcriptional factors AhR, Nrf2 and NF-κB probably involved in DOPAC enhanced ALDH activity; (7) As a low toxic food chemical, DOPAC impaired acetaldehyde-induced cytotoxicity. Taken together, these serious studies suggested both BITC and DOPAC showed enhanced ALDH activity which play important roles in prevention from acetaldehyde-induced liver damage possibly though transcriptional regulation of Nrf2 or AhR or NF-κB.

CHAPTER 2

Benzyl isothiocyanate ameliorates acetaldehyde-induced cytotoxicity by enhancing aldehyde dehydrogenase activity in murine hepatoma Hepa1c1c7 cells

2.1 Introduction

Alcohol, i.e., ethanol, is absorbed into the blood from the stomach to the small intestine, then distributed throughout the body. The metabolism of alcohol converting to acetic acid basically involves two enzymes; alcohol dehydrogenase converting ethanol to acetaldehyde and aldehyde dehydrogenase (ALDH) catalyzing the conversion from acetaldehyde to its corresponding acids (Matysiak-Budnik et al., 1996). Nineteen genes have already been identified as members of the human ALDH family (Vasiliou and Nebert, 2005). Among the ALDH family enzymes, ALDH2 is a mitochondrial enzyme ubiquitously expressed with the highest level in the liver and plays a pivotal role in the acetaldehyde detoxification (Yoshida et al., 1998). In Asian countries, about 40% of the people have the mutation of ALDH2, which is called ALDH2*2. The acetaldehyde-metabolizing ability of the ALDH2*2 individuals is only about 15% as efficient as that of wild type individuals. This mutation leads to the accumulation of acetaldehyde which causes significant damage to the liver and serious abnormal reactions of the body like vasodilation and facial flushing (Goedde et al., 1980), (Agarwal et al., 1981), (Xiao et al., 1995). ALDH polymorphism may also result in an increased vulnerability to developing cancer (Wang et al., 2014). In addition to ALDH2, a cytosolic ALDH, ALDH1A1, has also been reported to express at a high level in the liver tissue of humans and play a major role in the acetaldehyde metabolism (Lind, et al., 2008). Another cytosolic ALDH, ALDH3A1, has been suggested to assist ALDH2 in the metabolism of acetaldehyde and ethanol in vivo, even though it exhibits a selective activity for oxidation of aromatic and medium-chain aldehydes (Chen, et al., 2015).

Therefore, the strategy for enhancement of the liver ALDH activities by the administration of food phytochemicals is most likely to prevent humans, who are intolerant to alcohol with a low ALDH activity, from not only an alcohol-induced abnormal reaction, but also chronic diseases including cancer.

A previous study demonstrated that the ALDH activity could be enhanced by the activation of the Kelch like-ECH-associated protein 1/nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/antioxidant response element (ARE) pathway (Nguyen et al., 2000), (Kensler et al., 2007), (Baird and Dinkova-Kostova, 2011). Nrf2 is the key factor to modulate the phase 2 drug-metabolizing enzyme activity, including glutathione *S*-transferases (GSTs), glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H: quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1) (Nakamura and Miyoshi, 2010). Evaluation of 20 inducers of the phase 2 enzymes belonging to various chemical classes, including isothiocyanates, triterpenoids, flavonoids and stilbenes, demonstrated that their induction potencies of ALDH were closely correlated with those for the NQO1 induction (Ushida and Talalay, 2013).

Isothiocyanates (ITCs), mainly derived from cruciferous vegetables, such as broccoli, wasabi (Japanese horseradish) and watercress, are highly effective for cancer chemoprevention *in vitro* and *in vivo* (Nakamura and Miyoshi, 2010). We previously demonstrated that benzyl ITC (BITC), an ingredient in papaya (Nakamura et al., 2007), induces phase 2 drug-metabolizing enzymes such as GSTs (Nakamura et al., 2000).

In the present study, we assessed BITC as a potential inducer of the total ALDH activity using murine hepatoma Hepa1c1c7 cells. We demonstrated that BITC enhances the ALDH gene expression as well as the cytosolic and mitochondrial ALDH activities. Experiments with Nrf2 siRNA indicated that BITC induces the transcriptional expression

of ALDH isozymes by causing Nrf2 to accumulate in the nucleus. Furthermore, the pretreatment of BITC increased the resistance to the acetaldehyde-induced cytotoxicity.

2.2 Materials and Methods

2.2.1. Chemicals and antibodies

BITC was purchased from LKT laboratories, Inc. (St. Paul, MN, USA). Antibodies against ALDH2, Nrf2, lamin B1, and actin, siRNA for Nrf2 (Catalog No. sc-37049), siRNA for ALDH3A1 (Catalog No. sc-72033), and control scrambled siRNA (Catalog No. sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ALDH1A1 and ALDH3A1 were purchased from Cell Signaling Technology (Beverly, MA, USA) and ABCAM (Cambridge, MA, USA), respectively. α-Minimum essential medium (α-MEM), Lipofectamine® 3000 Transfection Kit, and Trizol reagent were purchased from Life Technologies (Carlsbad, CA, USA). Fatal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). Chemi-lumi One Super was purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Immobilon-P membrane was purchased from Merck Millipore (Billerica, MA, USA). The Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ReverTra Ace was purchased from TOYOBO Co., Ltd. (Osaka, Japan). Taq polymerase was purchased from Takara Bio, Inc. (Kusatsu, Japan). Pierce™ BCA Protein Assay Kit was purchased from Thermo Scientific (Meridian Rd., Rockford. USA). β-Nicotinamide-adenine dinucleotide, oxidized form (NAD^+) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2.2. Cell culture

Hepa1c1c7 cells and human hepatocellular carcinoma HepG2 cells were obtained from the American Type Culture Collection. Hepa1c1c7 cells and HepG2 cells were

grown at 37ºC in a 5% CO_2 atmosphere in α-MEM supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. RL34 cells were obtained from the Health Science Research Resources Bank, Osaka, Japan. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), and 0.37% $NaHCO_3$ at 37 °C.

2.2.3. Assay of ALDH activity

Hepa1c1c7, RL34 and HepG2 cells (1.2×10^6) were seeded on 60-mm dishes at 37ºC and 5% CO_2 atmosphere for 24 h, then treated with or without the different concentrations of BITC in 0.2% dimethyl sulfoxide (DMSO). The ALDH activity was measured as previously described by Moreb *et al.* (Moreb et al., 2000). Briefly, the cell lysates were prepared by 1 ml lysis buffer (50 mM Tris (pH 8), 25 mM EDTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% sarcosyl) and centrifugated at 10,000 rpm for 10 min at 4ºC. Twenty µl of 5 mM NAD^+ and 20 µl of 5 mM propionaldehyde (substrate) were added to 200 µl of supernatant to start this assay. NADH was measured by the change in absorbance at 340 nm over 5 min. One unit was defined as the amount of enzyme activity that catalyzed 1 µmol NAD^+ to NADH per minute. The mitochondrial and cytosolic/microsomal ALDH activities were separately measured according to the method of Ushida and Talalay (Ushida and Talalay, 2013). Briefly, lysis buffer contained 0.25 M sucrose, 1 mM 2-mercaptoethanol and 10 mM Tris–HCl (pH 7.4), cell lysates were homogenized and centrifuged at 750 g for 15 min. The supernatants were centrifuged twice at 12,000 g for 20 min to obtain the cytosolic/microsomal fractions and the mitochondrial fractions. The mitochondrial fractions (sediments) were dissolved in 0.25 M sucrose, diluted with one-half volume of

1% sodium bicarbonate containing 1% sodium deoxycholate (w/v) and sonicated at 4°C for five 2-min periods before the assays.

2.2.4. RNA extraction and RT-PCR

Hepa1c1c7 cells (5.0×10^5) were precultured in a 6-well plate for 24 h, then treated with or without the different concentrations of BITC. The total RNA was extracted using Trizol reagent according to the manufacturer's manual. The total RNA was reverse transcribed to cDNA using ReverTra Ace. PCR amplification was then performed with Taq polymerase. The Primers used in the PCR amplification are as follows: mALDH1A1, (F) 5'-gACAggCTTTCCAgATTggCTC-3' and (R) 5'-AAgACTTTCCCACCATTgAgTgC-3'; mALDH2, (F) 5'-TgAAgACggTTACTgTTACTgTCAAAgTgC-3' and (R) 5'-AgTgTgTgTggCggTTTTTCTC-3'; mALDH3A1, (F) 5'-gATgCCCATTgTgTgTgTTCg-3' and (R) 5'-CCACCgCTTgATgTCTCTgC-3'; mNQO1, (F) 5'-TCgAAgAACTTTCAgTATCC-3' and (R) 5'-TgAAgAgAgTACATggACCC-3'; mHO-1, (F) 5'-ACATCgACAgCCCCACCAAgTTCAA-3' and 5'-CTgACgAAgTgACgCCATCTgTgAg-3'; mβ-actin, (F) 5'-gCTCTTTTCCAgCCTTCCTT-3' and (R) 5'-CTTCTgCATCCTgTCAgCAA-3'. The cycles and annealing temperatures used in the PCR amplification are as follows: mALDH1A1, 20 cycles, 57ºC (product size 142 bp); mALDH2, 20 cycles, 57ºC (product size 115 bp); mALDH3A1 (product size 138 bp), 25 cycles, 56ºC; mNQO1, 22 cycles, 55ºC (product size 290 bp); mHO-1, 26 cycles, 56ºC (product size 203 bp); mβ-actin , 16 cycles, 65ºC (product size 455 bp). The PCR product were separated on an agarose gel

(3% or 4%), stained with ethidium bromide, and visualized under UV light. The relative densities of the bands were measured using Image J Software Program.

2.2.5. Western blot analysis

The whole cell lysates were prepared in lysis buffer (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol (DTT), 10 mM NaF, 1 mM $NaVO_3$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% SDS, 1% Triton X-100) containing protease inhibitor cocktail and left on ice for 20 min. After the cell lysates were sonicated and centrifuged, the supernatant containing 30 μg of protein were prepared for SDS-PAGE. For separation of nuclear fraction, the cells were first suspended in buffer-1 (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM $NaVO_3$ and PMSF) containing protease inhibitor cocktail and left on ice for 15 min. After 0.4% NP-40 was added, the cell lysates were centrifuged at 500 g for 4 min. Sediments were washed 3 times by buffer-1 and suspended in buffer-2 (20 mM HEPES pH 7.9, 400 mM NaCl, 1mM EDTA, 1mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM $NaVO_3$ and 1 mM PVSF) containing protease inhibitor cocktail, kept on ice for 15 min, then centrifuged 18000 g for 20 min. The supernatant containing 10 μg protein was prepared for SDS-PAGE. After the SDS-PAGE purification, the proteins were electrophoretically transferred to the PVDF membranes. The bound antibody was visualized using a Chem-Lumi One Super. The relative densities of the bands were measured using Image J Software Program.

2.2.6. MTT assay

Hepa1c1c7 cells (2 x 10^5) were seeded in a 96-well plate and incubated for 24 h. After the pretreatment of a non-toxic concentration of BITC (1 μM) for 24 h, the cells were treated with different concentrations of acetaldehyde for 3 h. After the acetaldehyde stimulation, each well was exposed to the MTT solution for an additional 2 h. The MTT containing formazan crystals in living cells were solubilized in a mixture of HCl and 2-propanol, then measured at 570 nm by spectrophotometry. The cell viability results were expressed as percentages compared to each of the controls.

2.2.7. RNA interference

Hepa1c1c7 cells (2.0 x 10^5) were seeded in a 6-well plate and transfected with Nrf2 siRNA (Santa Cruz, Catalog No. sc-37049, 120 μmol) or the control scrambled siRNA (Santa Cruz, Catalog No. sc-37007, 120 μmol) using Lipofectamine® 3000 Reagent according to the manufacturer's instruction. After 48 h of transfection, the cells were treated with DMSO or 5 μM BITC for 24 h followed by Western blot, RT-PCR, MTT assay and ALDH activity assay.

2.2.8. Statistical analysis

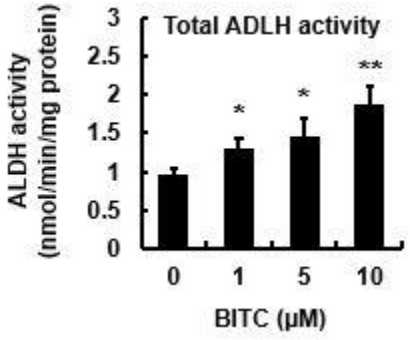
All values were expressed as means ± SD. Statistical significance was analyzed by Student's t-test or a one-way ANOVA followed by Tukey's HSD using XLSTAT software. A p-value of 0.05 was regarded to be statistically significant.

2.3. Results

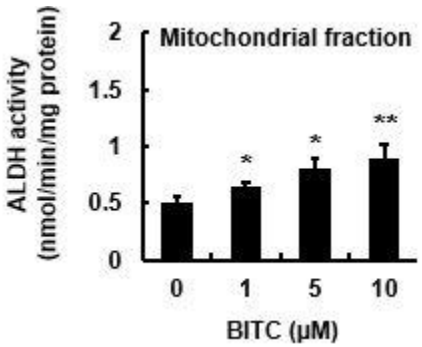
2.3.1. BITC enhanced the total ALDH enzyme activity

We initially determined the cytotoxic effect of BITC on Hepa1c1c7 cells and observed that the BITC at concentrations of more than 20 μM induced a significant toxicity (Fig. 2.5A). Therefore, 10 μM was used as the maximum concentration of BITC to test. As shown in Fig. 2.1A, we observed a significant increase in the total ALDH activity in the BITC-treated Hepa1c1c7 cells. When the cells were incubated with 10 μM BITC for 24 h, the total ALDH activity was enhanced about 2 times compared to the control group. We next separately investigated the ALDH activities in the cytosolic/microsomal fraction as well as in the mitochondrial fraction. The basal ALDH activity in the cytosolic/microsomal fraction was much higher than that in the mitochondrial fraction. As shown in Figs. 2.1B and 2.1C, the ALDH activities in each fraction were significantly increased by the treatment of 5 μM BITC for 24 h. Furthermore, as shown in Figs. 2.1D and 2.1E, BITC significantly increased the total ALDH activity not only in rat liver epithelial RL34 cells, but also in human hepatocellular carcinoma HepG2 cells. These results suggested that BITC is a potential enhancer of the total ALDH enzyme activity.

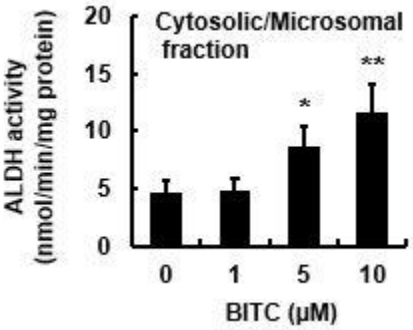
A



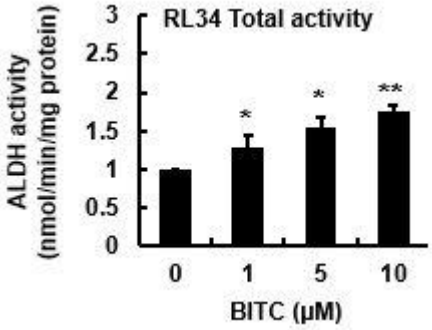
B


Mitochondrial fraction
ALDH activity
(nmol/min/mg protein)
2
1.5
1
0.5
0
*
*
**
0
1
5
10
BITC (μM)

C


Cytosolic/Microsomal
fraction
ALDH activity
(nmol/min/mg protein)
20
15
10
5
0
*
**
0
1
5
10
BITC (μM)

D


RL34 Total activity
ALDH activity
(nmol/min/mg protein)
3
2.5
2
1.5
1
0.5
0
*
*
**
0
1
5
10
BITC (μM)

E

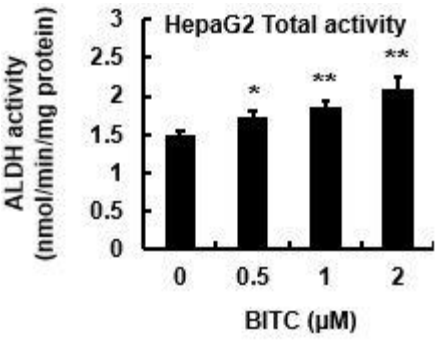


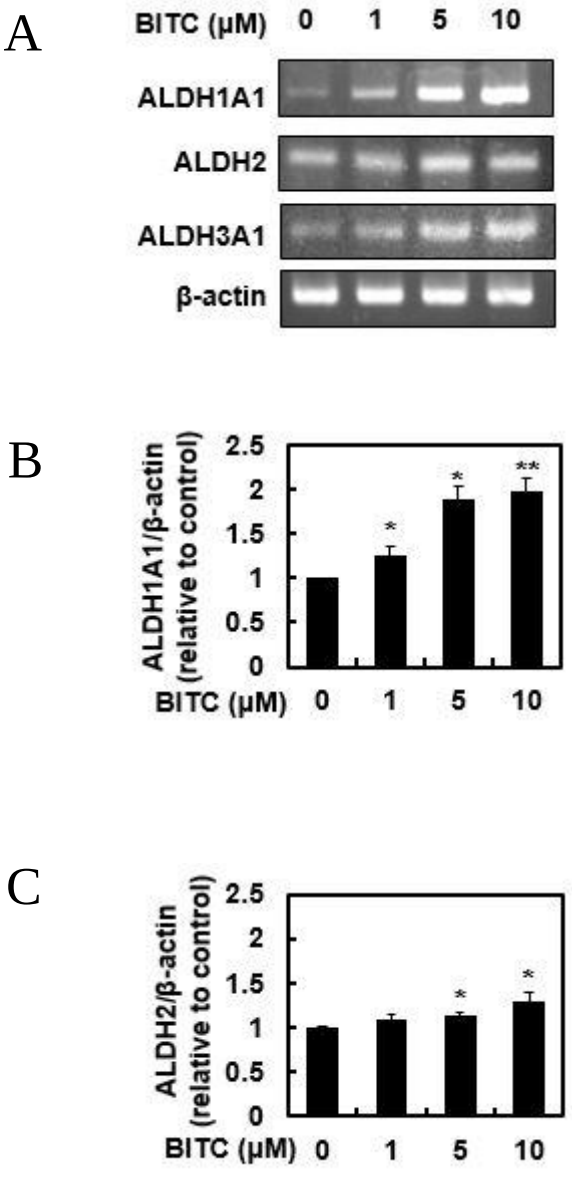
Fig. 2.1 Modulating effects of BITC on the ALDH activities in several hepatocyte cell lines.

Hepa1c1c7 cells or RL34 cells (1.2 x 10^6) were seeded on 60-mm dishes for 24 h, then treated with or without the different concentrations of BITC for 24 h. HepG2 cells (1.2 x 10^6) were seeded on 60-mm dishes for 24 h, then treated with or without the different concentrations of BITC for 3 h. The ALDH activity was measured using propionaldehyde as a substrate as previously described by Moreb et al. (Moreb et al., 2000). The mitochondrial and cytosolic/microsomal ALDH activities were separately measured according to the method of Ushida and Talalay (Ushida and Talalay, 2013). (A) total ALDH activity, (B) cytosolic/microsomal fractions and (C) mitochondrial fraction in Hepa1c1c7 cells, (D) total ALDH activity in RL34 cells, and (E) total ALDH activity in HepG2 cells. All values are expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to 0 μM BITC group; Student's t-test).

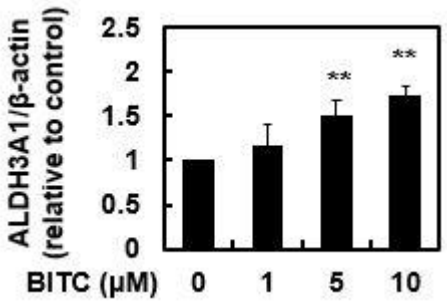
2.3.2. BITC enhanced the gene and protein expression of ALDHs

We next checked the gene expression of the classical family of ALDHs, such as ALDH1A1, ALDH2 and ALDH3A1, by RT-PCR. As shown in Figs. 2.2A, 2.2B, 2.2C and 2.2D BITC significantly increased each mRNA level of ALDH1A1, ALDH2 and

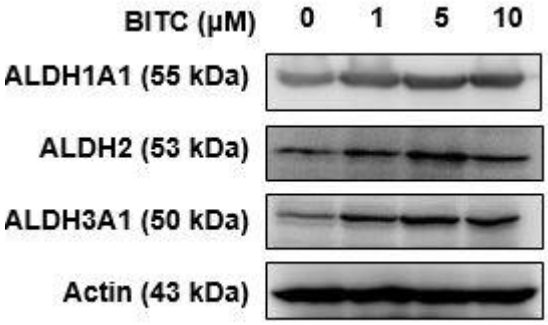
ALDH3A1 in a dose-dependent manner. Furthermore, as shown in Figs. 2.2E, 2.2F, 2.2G and 2.2H, BITC also significantly increased the protein levels of ALDH1A1, ALDH2 and ALDH3A1. These results suggested that BITC enhanced the total ALDH enzyme activity through a transcriptional regulation.



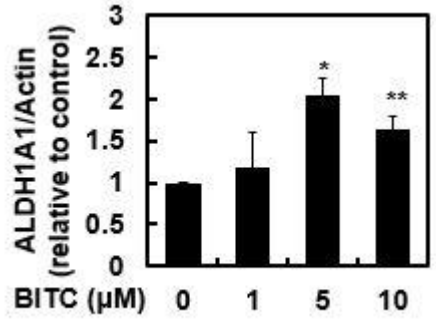
D


ALDH3A1/β-actin
(relative to control)
2.5
2
1.5
1
0.5
0
**
**
BITC (μM) 0 1 5 10

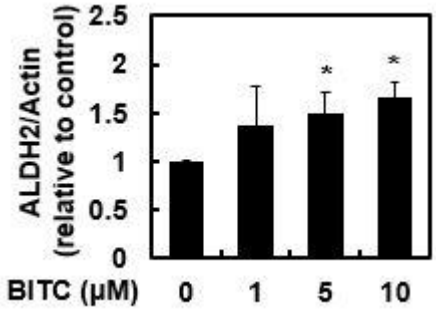
E


BITC (μM) 0 1 5 10
ALDH1A1 (55 kDa)
ALDH2 (53 kDa)
ALDH3A1 (50 kDa)
Actin (43 kDa)

F


ALDH1A1/Actin
(relative to control)
3
2.5
2
1.5
1
0.5
0
*
**
BITC (μM) 0 1 5 10

G


ALDH2/Actin
(relative to control)
2.5
2
1.5
1
0.5
0
*
*
BITC (μM) 0 1 5 10

H

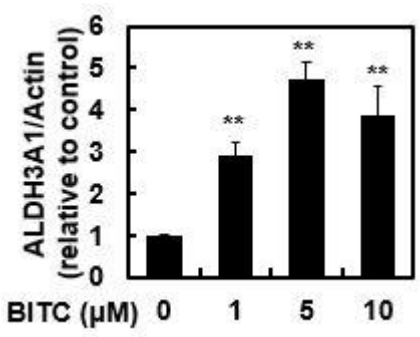


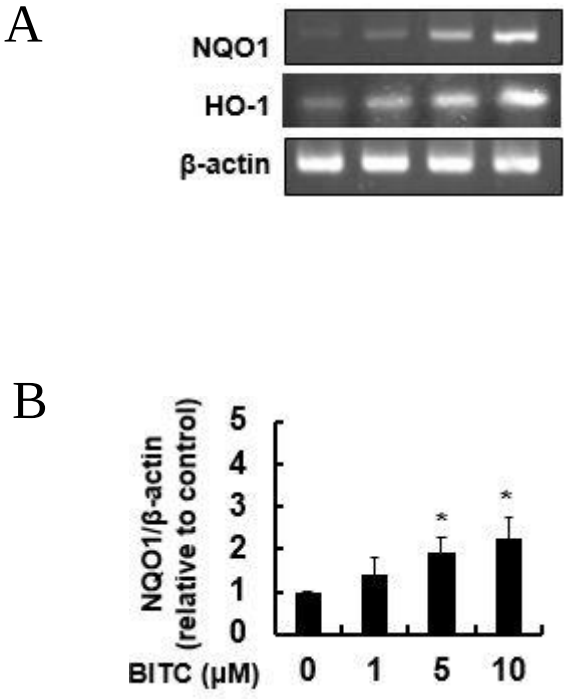
Fig. 2.2 Modulating effects of BITC on the gene and protein expressions of the classical family of ALDHs.

The total RNA was extracted from the Hepa1c1c7 cells treated with BITC for 24 h, then a RT-PCR analysis for each gene was carried out. (A) representative and quantitative data for (B) ADLH1A1, (C) ADLH2 and (D) ADLH3A1. The total protein was extracted from the Hepa1c1c7 cells treated with BITC for 24 h, then a Western blot analysis for each protein was carried out. (E) representative blots and quantitative data for (F) ADLH1A1, (G) ADLH2 and (H) ADLH3A1. All values are expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to 0 μM BITC group; Student's t-test).

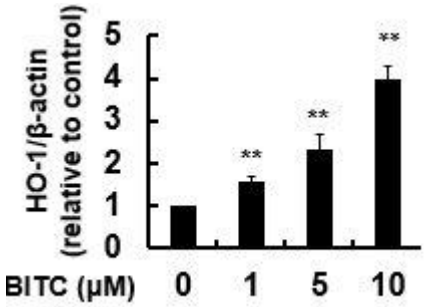
2.3.3. An Nrf2-dependent pathway was activated by BITC

Several ALDH genes have been identified as Nrf2-regulated ones by the microarray screening using Nrf2 knockout ($Nrf2^{-/-}$) mice (Thimmulappa et al., 2002). Nrf2 also controls the gene expression of the representative phase 2 detoxifying enzymes such as NQO1 and HO-1 (Li et al., 2014). As shown in Figs. 2.3A, 2.3B and 2.3C, the treatment of BITC for 24 h also significantly induced the gene expression of NQO1 and

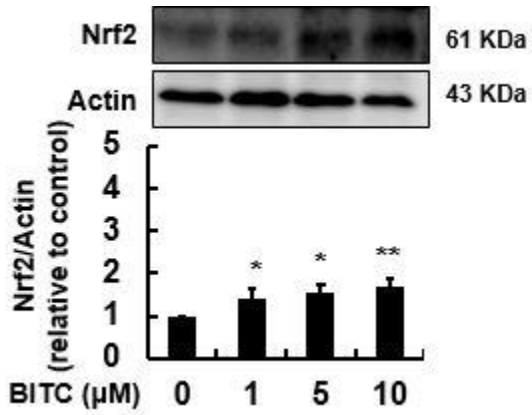
HO-1 in a dose-dependent manner. These results indicated that BITC might activate the Nrf2 pathway. Thus, we examined the Nrf2 protein expression and nuclear translocation by Western blotting. As shown in Fig. 2.3D, the treatment of BITC caused a significant increase in the Nrf2 level of the total cell lysate. Furthermore, the nuclear level of the Nrf2 protein was significantly increased by a 24-h BITC treatment (Fig. 2.3E). These results suggested that BITC actually activated the Nrf2 pathway, possibly through enhancement of the protein expression and nuclear translocation of Nrf2.



C


HO-1/β-actin
(relative to control)
5
4
3
2
1
0
**
**
**
BITC (μM) 0 1 5 10

D


Nrf2
61 KDa
Actin
43 KDa
Nrf2/Actin
(relative to control)
5
4
3
2
1
0
*
*
**
BITC (μM) 0 1 5 10

E

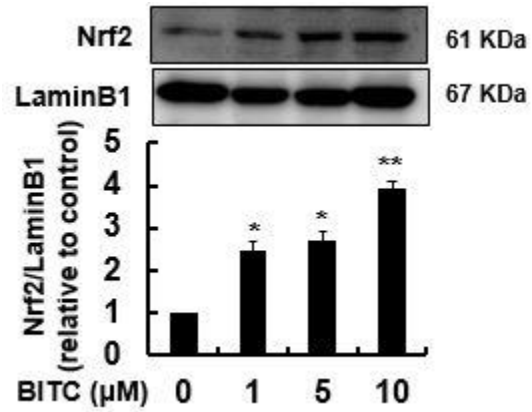

Nrf2
61 KDa
LaminB1
67 KDa
Nrf2/LaminB1
(relative to control)
5
4
3
2
1
0
*
*
**
BITC (μM) 0 1 5 10

Fig. 2.3 Modulating effects of BITC on the gene expression of the phase 2 enzymes and Nrf2 level in Hepa1c1c7 cells.

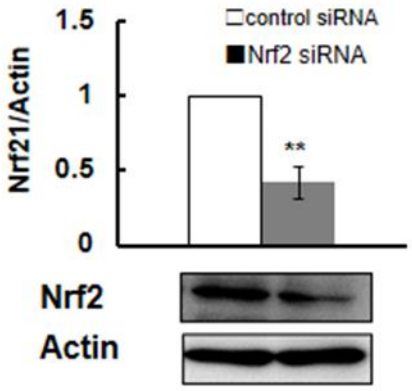
Hepa1c1c7 cells were treated with BITC for 24 h, then a RT-PCR analysis for each gene was carried out. (A) representative and quantitative data for (B) NQO1 and (C) HO-1. Hepa1c1c7 cells were treated with BITC for 24 h, and the nuclear fractions as well as total cell lysates were subjected to a Western blot analysis. (D) BITC increased the Nrf2 protein level in the whole lysate. (E) BITC increased the nuclear accumulation of Nrf2. All values are expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to 0 μM BITC group; Student's t-test)

2.3.4. Silencing Nrf2 affected the total ALDH activity

To confirm whether Nrf2 is involved in the BITC-enhanced ALDH activity, we used an siRNA technique to knockdown the Nrf2 protein expression. A Western blot analysis showed that the Nrf2 siRNA transfection significantly depleted the Nrf2 level to 40% compared to the control (Fig. 2.4A). When the cells were incubated with 5 μM BITC for 24 h, the nuclear level of Nrf2 was enhanced about 2.5 times compared to the control group (Fig. 2.4B), suggesting that the Nrf2 siRNA transfection under the present condition might be adequate for inhibition of the BITC-induced nuclear translocation. As shown in Fig 2.4B, both the basal and BITC-enhanced levels of the total ALDH activity were significantly decreased after knockdown of Nrf2. We next examined the effect of Nrf2 siRNA on the gene expression of ALDH1A1, ALDH2 and ALDH3A1. The basal expression of the ALDH1A1 gene was significantly decreased by the Nrf2 siRNA, whereas BITC still induced the ALDH1A1 gene expression even under the Nrf2 knockdown condition (Figs. 2.4C and 2.4D). Although the basal ALDH2 gene expression was not affected by the Nrf2 siRNA, the BITC-induced ALDH2 gene

expression significantly decreased (Figs. 2.4C and 2.4E). Finally, both the basal and inducible gene expressions of ALDH3A1 were significantly decreased by the Nrf2 siRNA (Figs. 2.4C and 2.4F). These results indicated that the Nrf2-dependent regulation of the expression of ALDH genes might concertedly contribute to not only the basal level, but also the BITC-induced enhancement of the total ALDH activity.

A



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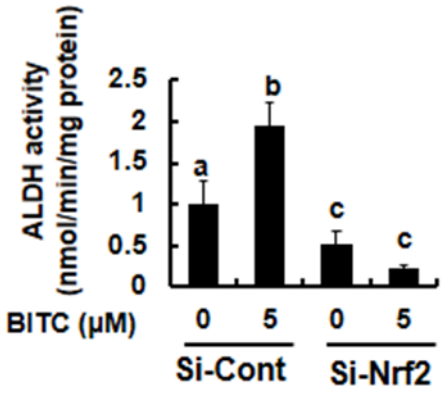
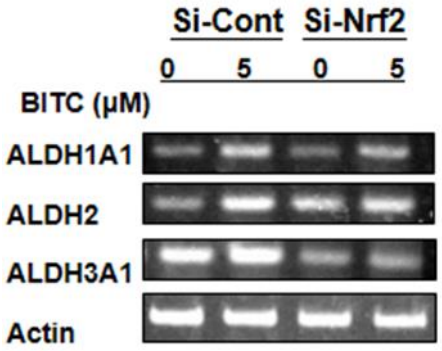
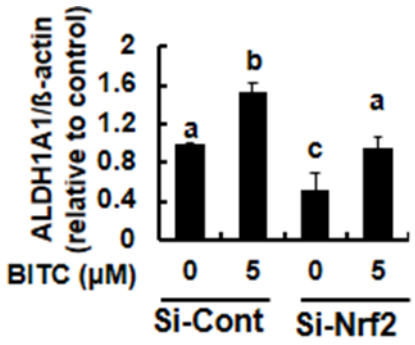


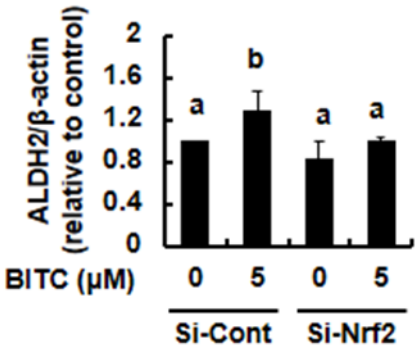
C


Si-Cont
Si-Nrf2
0
5
0
5
BITC (μM)
ALDH1A1
ALDH2
ALDH3A1
Actin

D


ALDH1A1/ß-actin
(relative to control)
2
1.6
1.2
0.8
0.4
0
a
b
c
a
BITC (μM)
0
5
0
5
Si-Cont
Si-Nrf2

E


ALDH2/β-actin
(relative to control)
2
1.6
1.2
0.8
0.4
0
a
b
a
a
BITC (μM)
0
5
0
5
Si-Cont
Si-Nrf2

F

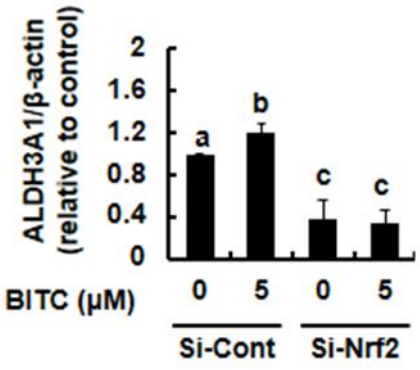

ALDH3A1/β-actin
(relative to control)
2
1.6
1.2
0.8
0.4
0
a
b
c
c
BITC (μM)
0
5
0
5
Si-Cont
Si-Nrf2

Fig. 2.4 Modulating effects of Nrf2 knockdown on the activity and gene expression of ALDHs.

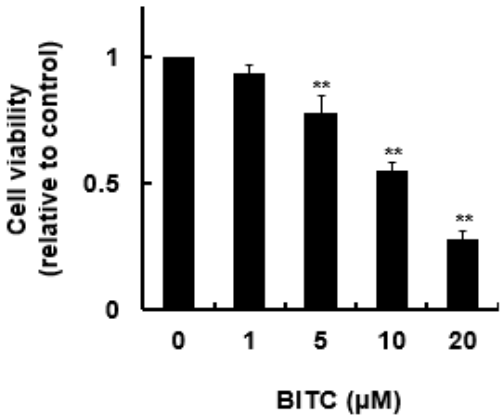
Hepa1c1c7 cells (2.0×10^5) were seeded at 6-well plates and transfected with the Nrf2 siRNA or control scrambled siRNA. After 48 h of transfection, the cells were treated with 5 μM BITC for 24 h followed by Western blot, RT-PCR, and ALDH activity assay. (A) The Nrf2 protein level in the whole cell lysates. (B) The basal and BITC-enhanced total ALDH activity. (C) Representative gene expression results. The basal and BITC-enhanced expression of (D) ALDH1A1, (E) ALDH2, and (F) ALDH3A1. All values are expressed as means ± SD of three separate experiments and analyzed by Student's t-test or a one-way ANOVA followed by Tukey's HSD using XLSTAT software. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

2.3.5. BITC pretreatment increased the resistance to the acetaldehyde-induced cytotoxicity

We finally examined the effect of BITC on the acetaldehyde-induced cytotoxicity. As shown in Fig. 2.5A, the treatment of BITC alone for 24 h significantly decreased the cell viability, however 1 μM BITC showed none toxicity. As shown in Fig. 2.5B, the treatment of acetaldehyde alone for 3 h significantly decreased the cell viability. Hepa1c1c7 cells were then pretreated with 1 μM BITC for 24 h before the acetaldehyde stimulation. As shown in Fig. 2.5B, the pretreatment of BITC for 24 h completely impaired the acetaldehyde-induced cytotoxicity. To determine whether the inhibition of the acetaldehyde cytotoxicity by BITC was regulated by the Nrf2 pathway, the effect of Nrf2 siRNA on the cytoprotection by BITC was examined. Nrf2 siRNA itself did not cause any cytotoxicity in Hepa1c1c7 cells (Fig. 2.5C). The siRNA-mediated depletion

of Nrf2 counteracted the protective effect of BITC on the acetaldehyde-induced cytotoxicity, as compared to the control siRNA (Fig. 2.5C). These results suggested that BITC mitigated the acetaldehyde cytotoxicity through the Nrf2 pathway. Finally, to determine whether the inhibition of the acetaldehyde cytotoxicity by BITC was regulated by ALDH3A1, the effect of ALDH3A1 siRNA on the cytoprotection by BITC was examined. As shown in Fig. 2.5D, the ALDH3A1 siRNA transfection significantly depleted the ALDH3A1 level to 20% compared to the control (Fig. 2.5D). Although ALDH3A1 siRNA itself did not cause any cytotoxicity in Hepa1c1c7 cells, it counteracted the protective effect of BITC on the acetaldehyde-induced cytotoxicity (Fig. 2.5E). These results suggested that BITC mitigated the acetaldehyde cytotoxicity, at least partly, through the ALDH3A1 up-regulation, even though the other ALDHs could not be ruled out in the mechanism.

A



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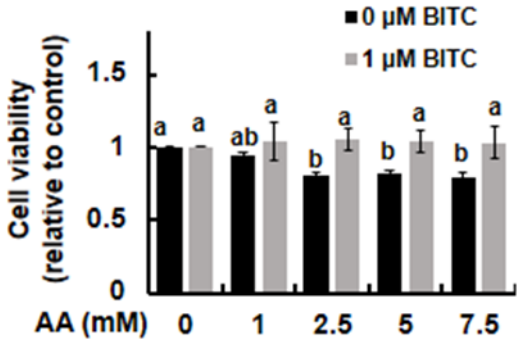

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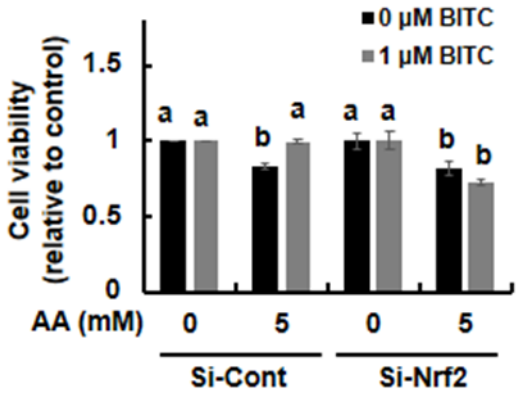
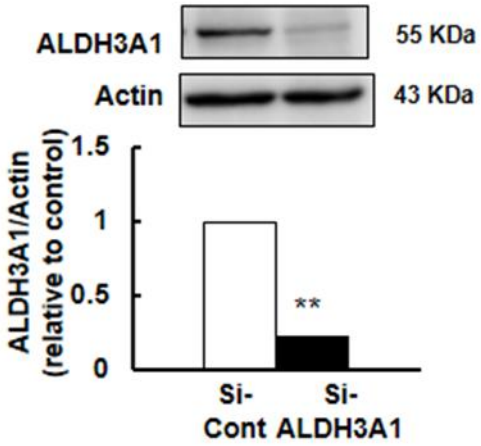


D



E

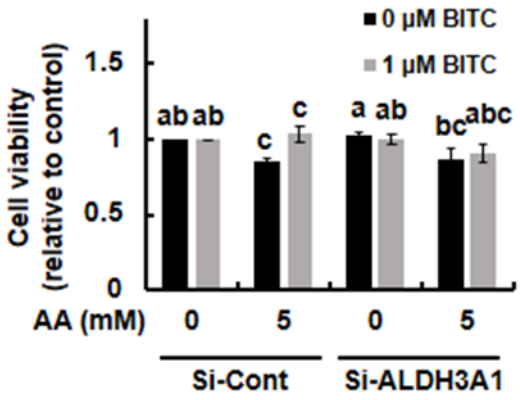


Fig. 2.5 Nrf2-dependent protection against acetaldehyde by BITC and ALDH3A1-dependent protection against acetaldehyde by BITC.

Hepa1c1c7 cells (2×10^5) were seeded on a 96-well plate and incubated for 24 h. After the pretreatment of 1 μM BITC for 24 h, the cells were treated with different concentrations of acetaldehyde for 3 h. After the acetaldehyde stimulation, an MTT assay was carried out. (A) Inhibitory effect of BITC treatment on the Hepa1c1c7 cells. (B) Inhibitory effect of BITC pretreatment on the acetaldehyde-induced cytotoxicity. (C) Impairment of BITC-enhanced resistance to the acetaldehyde-induced cytotoxicity by the Nrf2 siRNA. Hepa1c1c7 cells (2.0×10^5) were seeded at 6-well plates and transfected with the ALDH3A1 siRNA or control scrambled siRNA, then incubated for 48 h. (D) The ALDH3A1 protein level in the whole cell lysates. (E) Impairment of BITC-enhanced resistance to the acetaldehyde-induced cytotoxicity by the ALDH3A1 siRNA. All values are expressed as means ± SD of three separate experiments and analyzed by Student's t-test or a one-way ANOVA followed by Tukey's HSD using XLSTAT software. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

2.4. Discussion

In the present study, we demonstrated that BITC is a potential enhancer of the total ALDH enzyme activity. BITC dose-dependently enhanced not only the total ALDH activity, but also the cytoplasmic/mitochondrial ALDH activity for 24 h (Fig. 2.1). Simultaneously, the BITC treatments increased the gene expression of not only mitochondrial ALDH, ALDH2, but also cytosolic ALDHs, ALDH1A1, and ALDH3A1 (Fig. 2.2). These results suggested that BITC enhanced the total ALDH activity through a transcriptional regulation. Ushida *et al.* reported that other ITCs, such as sulforaphane, erucin, ilberin and 4-(rhamnopyranosyloxy)-benzyl isothiocyanate, have potencies for induction of the ALDH activity between 2-20 μM (Ushida and Talalay, 2013), whereas phenethyl ITC was reported to be a potential inhibitor of the ALDH activity (Lindros et al., 1995). A food component, alpha-lipoic acid, has been found to significantly increase the ALDH2 activity in isolated rat heart mitochondria at a concentration of more than 100 μM (Wenzel et al., 2007). Four flavonoids from *Echinosophora koreensis* also have a positive effect on the ALDH activity *in vivo* (Choi et al., 2009). Compared to the other ALDH enhancers, BITC is one of the most potent food components for increasing the total ALDH activity.

BITC significantly enhanced the gene expression of the Nrf2-dependent genes, such as NQO1 and HO-1 (Fig. 2.3), as well as the ALDH genes (Fig. 2.2). Nrf2 is a key transcriptional factor which activates the expression of the genes that contain ARE in their promoter. Nrf2 translocates to the nucleus where it binds to the ARE sequence, associated with its target genes and proteins. We confirmed that BITC increased both the protein expression and its nuclear translocation of Nrf2 (Figs. 2.3D and 2.3E). Furthermore, the pretreatment of the Nrf2 siRNA decreased both the basal and BITC-enhanced total ALDH activities, suggesting that the BITC-enhanced ALDH

activity is regulated by the Nrf2-dependent pathway. However, the Nrf2-dependency was varied between the basal and BITC-induced expressions of ALDH1A1, ALDH2 and ALDH3A1; the ALDH1A1 gene required Nrf2 for its basal, but not the BITC-induced expression; ALDH2 only required Nrf2 for the BITC-induced expression and; ALDH3A1 required Nrf2 for both its basal and BITC-induced expressions. These results suggested that the Nrf2-dependent pathway is, at least partly, involved in the BITC-induced gene expression of ALDHs. In addition to the Nrf2 dependent pathway, other signing pathways are also postulated. The inhibition of the STAT3 signaling pathway or its activator EZH2 genetically or pharmacologically diminished the level of the ALDH(+) cells and clonogenicity (Shao et al., 2014). AMP-activated protein kinase regulates the ALDH1A1 and ALDH2 expressions (Choi et al., 2011) (Kiefer et al., 2012). The gene expression of ALDH3s was shown to be mediated by an aryl hydrocarbon receptor- (Sládek, 2003) and NF-κB-dependent pathway (Weiner, 2007). The retinoic acid response element is responsible for down-regulation of ALDH1A1 through interaction of the retinoic acid receptor and CCAAT/enhancer binding protein β (Elizondo, et al., 2000). Even though other signaling pathways might be involved in the BITC-enhanced gene expression of ALDH1A1 and ALDH2, Nrf2 is one of the key transcriptional factors for the BITC-enhanced ALDH activity.

The cytotoxic effect of acetaldehyde has also been reported in various cellular models which are derived from the liver (Kurose et al., 1997), gastrointestinal tract (Rao, 1998), gonads (Mapoles et al., 1994), immune system (Sisson and Tuma, 1994) and brain (Yan et al., 2016). We also confirmed that acetaldehyde dose-dependently induced a cytotoxicity in mouse hepatoma Hepa1c1c7 cells (Fig. 2.5B). Tanaka *et al.* found that acetaldehyde triggered oxidative stress via mitochondrial superoxide production in the esophageal keratinocytes and that ALDH2-deficient cells were highly susceptible to the ethanol- or acetaldehyde-mediated toxicity (Tanaka et al., 2016). $ALDH2^{-/-}$ mice are

more susceptible to the ethanol and acetaldehyde-induced toxicity than the wild type mice (Yu et al., 2009). Because the ALDH status is responsible for the sensitivity of the acetaldehyde-induced toxicity, we examined the effect of BITC on the acetaldehyde-induced cytotoxicity. BITC (1 μM) actually impaired the acetaldehyde cytotoxicity (Fig. 2.5B). However, BITC did not impair the acetaldehyde-induced cytotoxicity in the Nrf2-knockdown cells as well as in the ALDH3A1-knockdown cells (Fig. 2.5C and 2.5E). These findings suggested that the inhibitory effect of BITC on the acetaldehyde-induced cytotoxicity is controlled by the Nrf2-dependent pathway and, at least in part, ALDH3A1 activity. The present data are consistent with a previous study showing that $Nrf2^{-/-}$ mice underwent a significant reduction in their ability to detoxify acetaldehyde (Lamlé et al., 2008). Anni *et al.* suggested another plausible mechanism for impairment of the acetaldehyde-induced toxicity which involves cysteine and glutathione (Anni et al., 2003). BITC not only induced the gene expression of ALDHs, but also the phase 2 genes including HO-1 (Fig. 4B), GST (Nakamura et al., 2000) and GCLC (Miyoshi et al., 2008). All of the HO-1, GST and GCLC genes have been reported to play an effective role in protection against oxidative stress (Ghattas et al., 2002) (Zou et al., 2014) (Franklin et al., 2002). Therefore, the BITC-induced phase 2 enzymes cannot be ruled out in the beneficial effect on amelioration of the acetaldehyde-induced cytotoxicity.

The present study demonstrated that BITC significantly increased the total ALDH enzyme activity and the gene expression of ALDHs dependent on the Nrf2 pathway. BITC mitigated the acetaldehyde-induced cytotoxicity which is also Nrf2-dependent. The present study represents a potentially efficient strategy to prevent the alcohol-induced abnormal reaction.

CHAPTER 3

3,4-Dihydroxyphenylacetic acid is a potential aldehyde dehydrogenase inducer in murine hepatoma Hepa1c1c7 cells

3.1 Introduction

3,4-Dihydroxyphenylacetic acid (DOPAC) is one of the major colonic microflora-produced catabolites of quercetin glycosides, such as quercetin 4′-glucoside (Q4′G) (Mullen et al., 2008), rutin (Ura et al., 2002), and hyperoside (quercetin 3-galactoside)(Yang et al., 2013). Human fecal bacteria have an ability to catalyze the formation of DOPAC(Peng et al., 2014) and an excretion of DOPAC was increased in human urine after the digestion of polyphenols from chocolate (Rios et al., 2003). DOPAC has also been identified as a significant metabolite of rutin in human blood by gas chromatography-mass spectrometry (Sawai et al., 1987). DOPAC is also known as a metabolite of a neurotransmitter dopamine. Taken together, the actual occurrence of DOPAC in humans is strongly suggested (Gesi et al., 2008). We have recently identified DOPAC as a predominant antioxidative catabolite of quercetin glycosides (Tang et al., 2016). A pulldown assay using a DOPAC click chemistry probe identified Kelch-like ECH-associated protein 1 and aryl hydrocarbon receptor (AhR) as the potential binding target proteins for the phase 2 drug-metabolizing enzyme up-regulation (Nakashima et al., 2016). DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity in hepatocytes (Tang et al., 2016). In addition to its antioxidant-related activities, DOPAC mitigated the pro-inflammatory cytokine secretion from peripheral blood mononuclear cells by in lipopolysaccharide (Monagas et al., 2009) and the P-selectin expression in platelets (Rechner and Kroner, 2005).

The physiological metabolism from ethanol into acetic acid basically involves two enzymes, alcohol dehydrogenase and aldehyde dehydrogenase (ALDH)

(Matysiak-Budnik et al., 1996). The mutation of certain ALDH genes or ALDH polymorphism results in the enhanced acetaldehyde accumulation, which causes serious damage to the liver and abnormal reaction like vasodilation and facial flushing (Jin et al., n.d.)(Abe et al., 2015). Therefore, the strategy for enhancement of liver ALDH activities by the consumption of food phytochemicals is most likely to prevent humans from an alcohol-induced abnormal reaction.

In this study, to explore the possibility of DOPAC as a potential enhancer of the ALDH activity, we examined the protective effect of DOPAC on the acetaldehyde-induced cytotoxicity *in vitro*. Our results showed that DOPAC increased the total ALDH activity as well as nuclear protein levels of the transcriptional factor Nrf2 and AhR. Furthermore, the pretreatment of DOPAC increased the resistance to the acetaldehyde-induced cytotoxicity.

3.2 Materials and Methods

3.2.1. Chemicals and antibodies

DOPAC was obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies against AhR, Nuclear factor κB (NF-κB), and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Nrf2 were purchased from Cell signaling Technology, Inc**.** (Beverly, MA, USA). Other are as shown in Chapter 2.

3.2.2 Cell culture and treatment

The mouse hepatoma cell line Hepa1c1c7 from the American Type Culture Collection were grown at 37^{o}C in a 5% CO_2 atmosphere in α-MEM supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Hepa1c1c7 cells (1.2 x 10^6) were cultured on 60-mm dishes at 37^{o}C and 5% CO_2 atmosphere for 24 h and then treated with or without the different concentrations of DOPAC in 0.2% dimethyl sulfoxide (DMSO) for 6 h.

3.2.3 MTT assay

See Chapter 2.

3.2.4 Western blotting assay

See Chapter 2.

3.2.5 ALDH activity assay

See Chapter 2.

3.3 Results

3.3.1 DOPAC enhanced ALDH activity

As shown in Fig. 3.1, DOPAC significantly and concentration-dependently enhanced the total ALDH activity after 6 h treatment. The total ALDH activity in the cells treated with 10 μM DOPAC, the minimal concentration for significant enhancement, was 1.7-fold greater than that of control.

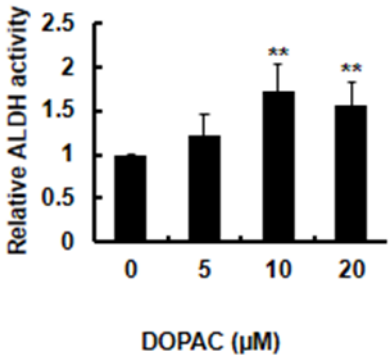


Fig.3.1 Modulating effects of DOPAC on the enzyme activity.

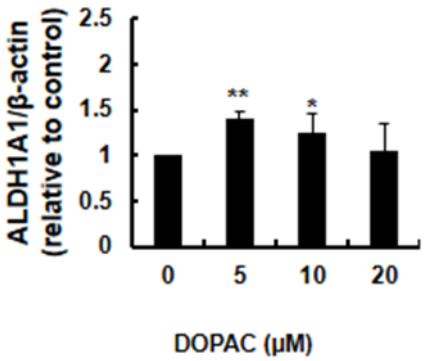
Hepa1c1c7 cells were treated with the indicated concentrations of DOPAC for 6 h. The ALDH activity was measured using propionaldehyde as a substrate. All values are expressed as means ± SD of three separate experiments (*p <0.05, **p <0.01 compared to 0 μM DOPAC group; Student's t-test)

3.3.2 Effect of DOPAC on gene expression of ALDHs

The ALDHs consist of a large family of enzymes catalyzing the conversion of different aldehydes into their corresponding acids. Among 19 genes in ALDH family, ALDH1A1 and ALDH2 has been reported to express at a high level in liver tissue of human and plays a major role in acetaldehyde metabolism (Lind et al., 2008). Among the ALDH family, ALDH2 is a main member catalyzing ethanol metabolite acetaldehyde

into acetic acid (Agarwal et al., 1981). ALDH3A1 has also been suggested to assist ALDH2 in the metabolism of acetaldehyde and ethanol *in vivo* (Chen et al., 2015). Thus we examined the effect of DOPAC on the gene expression of ALDH1A1, ALDH2 and ALDH3A1. As shown in Figs. 3.2 (A),3.2 (B) and 3.2 (C), DOPAC significantly increased each gene expression of ALDH1A1, ALDH2 and ALDH3A1. Therefore, DOPAC acts as a potential inducer of the total ALDH activity, possibly through the transcriptional regulation of not only ALDH2, but also ALDH1A1 and ALDH3A1.

A



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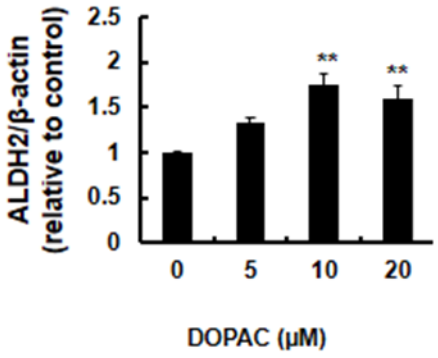

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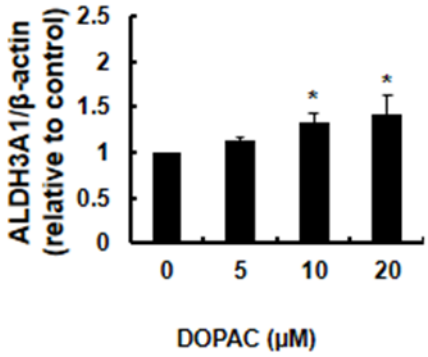


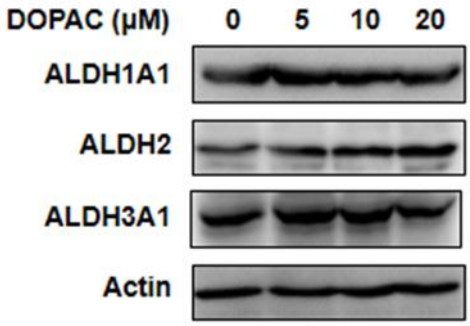
Fig. 3.2 Modulating effects of DOPAC on gene expression of ALDHs.

Hepa1c1c7 cells were treated with the indicated concentrations of DOPAC for 6 h. (A) A RT-PCR analysis for each gene was carried out; (B) ALDH1A1, (C) ALDH2, and (D) ALDH3A1. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to DOPAC 0 μM group, Student's t-test).

3.3.3 Effect of DOPAC on protein expression of ALDHs

We next checked the protein expression of the classical family of ALDHs, such as ALDH1A1, ALDH2 and ALDH3A1, by Western blotting. As shown in Fig. 3.3, DOPAC significantly increased the protein levels of ALDH1A1, ALDH2 and ALDH3A1. These inductions of protein expression probably because their gene expressions were increased.

A



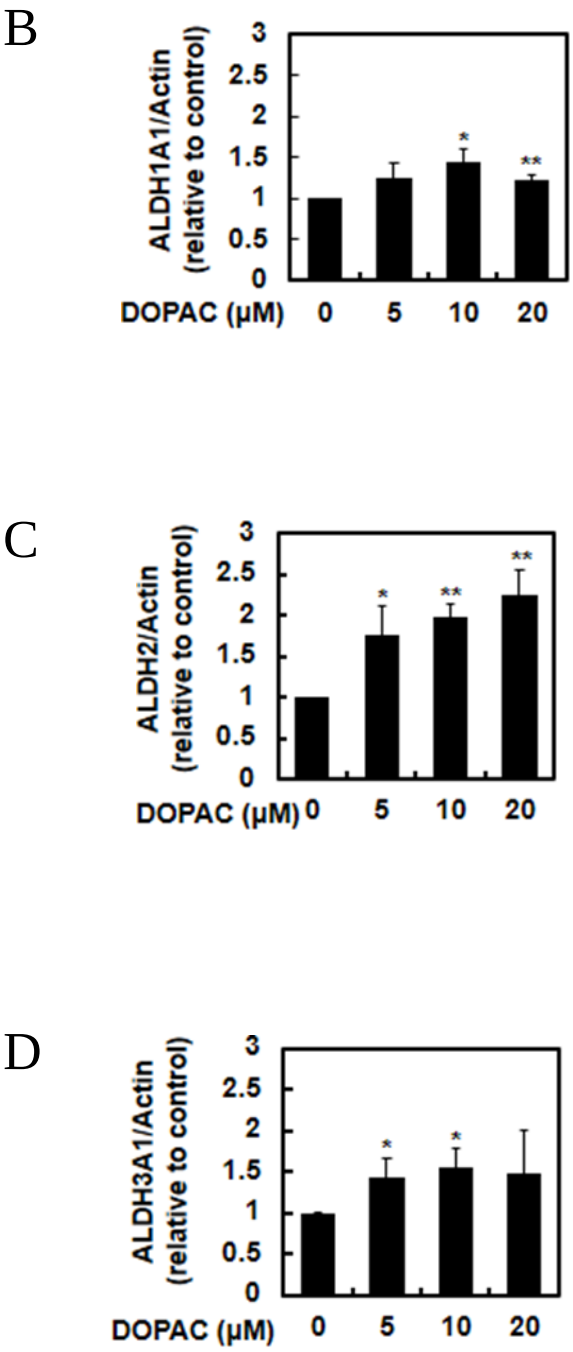


Fig. 3.3 Modulating effects of DOPAC on the protein expression of ALDHs.

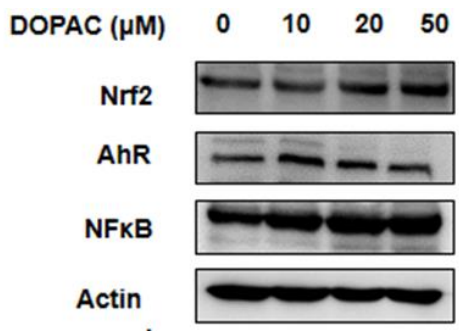
Hepa1c1c7 cells were treated with the indicated concentrations of DOPAC for 6 h. A Western blotting analysis for each protein was carried out; (A) Representative protein

expression results. (B) ALDH1A1, (C) ALDH2, and (D) ALDH3A1. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to DOPAC 0 μM group, Student's t-test).

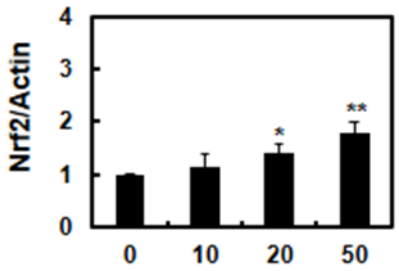
3.3.4 Effect of DOPAC on protein level of Transcriptional factor Nrf2, AhR and NF-κB.

Previous study using a DOPAC click chemistry probe indicated that the signaling pathway of Nrf2 and/or AhR is attributable to the gene expression of phase 2 drug-metabolizing enzymes (Nakashima et al., 2016). NF-κB is known as the nuclear factor interacted positively or negatively with Nrf2 and AhR (Wakabayashi et al., 2010). To investigate the possibility whether transcriptional factors, such as Nrf2, AhR and NF-κB, are involved in the ALDH expression, we examined their protein expression. As shown in Fig. 3.4A, 3.4B, 3.4C and 3.4D, DOPAC at 10 and 20 μM significantly increased the protein expression of Nrf2 and AhR, but not that of NF-κB. Next we checked the nuclear translocation of these transcriptional factors. The nuclear levels of Nrf2 and AhR significantly increased after the DOPAC treatment, whereas the nuclear level of NF-κB was attenuated by the DOPAC treatement (Fig. 3.4E, 3.4F, 3.4G and 3.4H). These results suggested that DOPAC is able to activate the signaling pathways of both Nrf2 and AhR, but rather inhibit the NF-κB pathway.

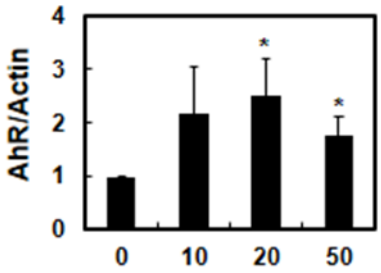
A


DOPAC (μM)
0
10
20
50
Nrf2
AhR
NFκB
Actin

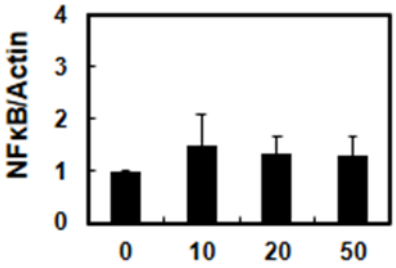
B


Nrf2/Actin
4
3
2
1
0
*
**
0
10
20
50

C


AhR/Actin
4
3
2
1
0
*
*
0
10
20
50

D


NFκB/Actin
4
3
2
1
0
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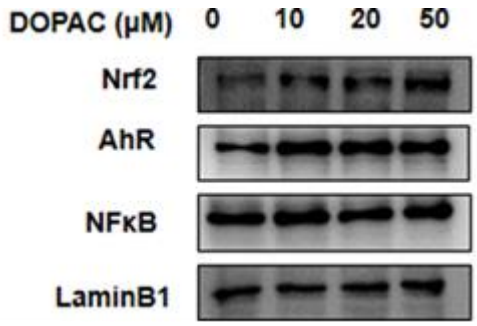
10
20
50

E



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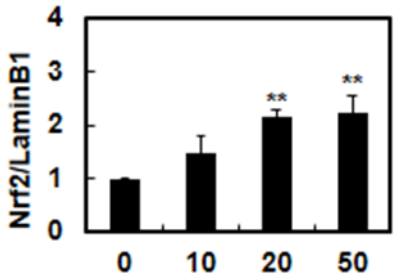
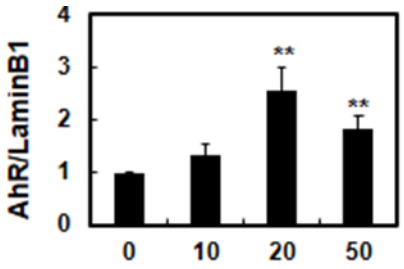


G



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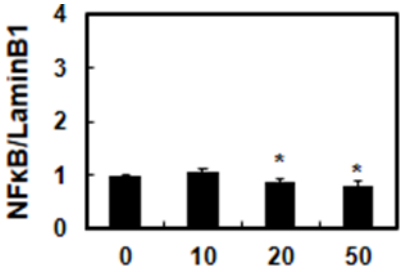


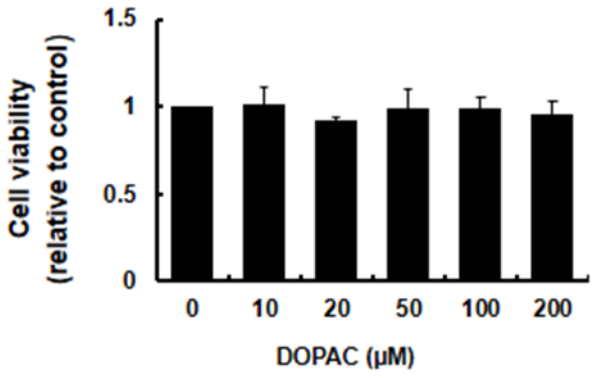
Fig. 3.4 Modulating effects of DOPAC on the protein expression and nuclear translocation of Nrf2, AhR and NF-κB.

Hepa1c1c7 cells were treated with DOPAC for 6 h, and nuclear fractions as well as total cell lysates were subjected to a Western blot analysis. The protein levels of (A) Representative results, (B) Nrf2, (C) AhR and (D) NF-κB in the whole lysate. The nuclear accumulation of (E) Representative results, (F) Nrf2, (G) AhR and (H) NF-κB. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to DOPAC 0 μM group, Student's t-test).

3.3.5 DOPAC mitigated acetaldehyde-induced cytotoxicity

Acetaldehyde, the primary metabolite of ethanol in human body, covalently binds to a variety of thiols, such as GSH and protein sulfhydryls, and consequently induces lipid peroxidation, thereby altering the liver function and structure (Matysiak-Budnik et al., 1996). We finally examined the effect of the DOPAC pretreatment on the acetaldehyde-induced cytotoxicity. Although the 6-h treatment of DOPAC at concentrations up to 200 μM did not significantly influence the cell viability (Fig. 3.5A), the 3-h incubation with acetaldehyde at each concentration significantly decreased the cell viability (Fig. 3.5B). The pretreatment of 10 μM DOPAC for 6 h completely impaired the acetaldehyde-induced cytotoxicity (Fig.3.5B), suggesting that DOPAC at the concentration required for the inducible expression of the ALDHs actually exhibited a cytoprotective effect.

A



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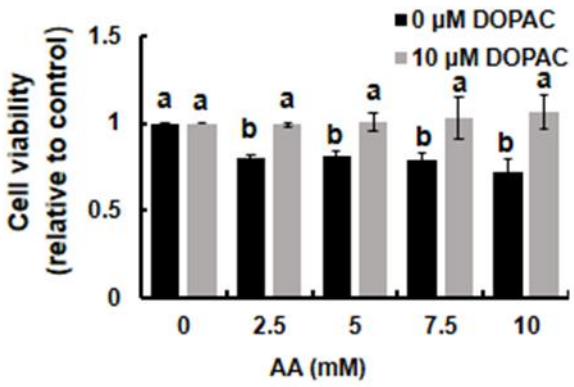

Fig. 3.5 Mitigating effect of DOPAC on the acetaldehyde-induced cytotoxicity in Hepa1c1c7 cells.

Hepa1c1c7 cells (2 x 10^5) were seeded in a 96-well plate and incubated for 24 h. (A) Effect of DOPAC on cell viability of Hepa1c1c7 cells. Cells were treated with different concentrations of DOPAC for 6 h. (B) Inhibitory effect of DOPAC pretreatment on the acetaldehyde-induced cytotoxicity. After the pretreatment of 10 μM DOPAC for 6 h, the cells were treated with different concentrations of acetaldehyde for 3 h. After the acetaldehyde stimulation, an MTT assay was carried out. All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

3.4 Discussion

As shown in Fig. 3.1A, DOPAC significantly and concentration-dependently enhanced the total ALDH activity after a 6-h treatment. The total ALDH activity in the cells treated with 10 μM DOPAC, the minimal concentration for a significant enhancement, was 1.7-fold greater than that of the control. The ALDHs consist of a large family of enzymes catalyzing the conversion of different aldehydes into their corresponding acids. Among the 19 genes in the ALDH family, ALDH1A1 and ALDH2 have been reported to express at a high level in the liver tissue of humans and play a major role in the acetaldehyde metabolism (Yoshida et al., 1998)(Lind et al., 2008)(Agarwal et al., 1981). ALDH3A1 has also been suggested to assist ALDH2 in the metabolism of acetaldehyde and ethanol in vivo (Chen et al., 2015). Thus we examined the effect of DOPAC on the gene expression of ALDH1A1, ALDH2 and ALDH3A1. As shown in Figs. 3.2A, 3.2B and 3.2C, DOPAC significantly increased each gene expression of ALDH1A1, ALDH2 and ALDH3A1, respectively. Furthermore, as shown in Fig. 3.3, DOPAC also significantly increased the protein levels of ALDH1A1, ALDH2 and ALDH3A1. Therefore, DOPAC acts as a potential inducer of the total ALDH activity, possibly through the transcriptional regulation of not only ALDH2 and ALDH1A1, but also ALDH3A1.

A previous study using a DOPAC click chemistry probe indicated that the signaling pathway of Nrf2 and/or AhR is attributable to the gene expression of the phase 2 drug-metabolizing enzymes (Nakashima et al., 2016)(Tang et al., 2016). NF-κB is known as the nuclear factor that interacts positively or negatively with Nrf2 and AhR (Wakabayashi et al., 2010). To investigate whether Nrf2, AhR and NF-κB, are involved in the ALDH expression, we examined their protein expression. As shown in Fig. 3.4, DOPAC showed the tendency to increase the total protein expression of Nrf2 and AhR,

but not NF-κB. We next checked the nuclear translocation of these transcriptional factors. The nuclear levels of Nrf2 and AhR significantly increased after the DOPAC treatment, whereas the nuclear level of NF-κB was attenuated by the DOPAC treatment (Figs. 3.4E, 3.4F, 3.5G and 3.5H). These results suggested that DOPAC is able to activate the signaling pathways of both Nrf2 and AhR, but rather inhibit the NF-κB pathway.

Acetaldehyde, the primary metabolite of ethanol in human body, covalently binds to a variety of thiols, such as GSH and protein sulfhydryls, and consequently induces lipid peroxidation, thereby altering the liver function and structure (Matysiak-Budnik et al., 1996). We finally examined the effect of the DOPAC pretreatment on the acetaldehyde-induced cytotoxicity. Although the 6-h treatment of DOPAC at concentrations up to 200 μM did not significantly influence the cell viability (Fig. 3.5A), the 3-h incubation with acetaldehyde at each concentration significantly decreased the cell viability (Fig. 3.5B). The pretreatment of 10 μM DOPAC for 6 h completely impaired the acetaldehyde-induced cytotoxicity (Fig. 3.5B), suggesting that DOPAC at the concentration required for the inducible expression of the ALDHs actually exhibited a cytoprotective effect.

Nrf2 has been reported as a transcriptional factor involved in the inducible expression of ALDH1 (Wu et al., 2012), ALDH2 (Nair et al., 2006), and ALDH3A1 (Sládek, 2003). In this study, we observed that DOPAC significantly increased the nuclear translocation of Nrf2 (Figs. 3.4B and 3.4F). A previous study demonstrated that the knockdown of AhR reduced the total ALDH activity by 80% in Hs578T cells (Bunaciu and Yen, 2011). The gene expression of ALDH3 was shown to be mediated by an AhR/xenobiotic response element axis (Sládek, 2003). AhR has been identified as a potential target of covalent modification by DOPAC (Nakashima et al., 2016).

Expectedly, DOPAC significantly increased the nuclear level of the AhR proteins (Figs. 3.4C and 3.4G). Although the promoter region of the ALDH3A1 gene is also reported to bind with NF-κB, DOPAC did not increase its nuclear level (Fig. 3.4D). Taken together, DOPAC might activate both the AhR and Nrf2-dependent pathways, but not the NF-κB pathway, to increase the expression of the ALDH genes. Keap1 is postulated as a plausible target of electrophiles that facilitate the depression of Nrf2 (Nakamura and Miyoshi, 2010). Electrophilic quinones have also been reported to have capability to covalently bind to and activate AhR (Abiko et al., 2015). Our group have successfully detected the direct modification of Keap1 and AhR by DOPAC using its click chemistry probe and the pull down assay (Nakashima et al., 2016). These results indicated that covalent modification of Keap1 and AhR by DOPAC might be involved in the activation of these pathways and up-regulation of ALDH genes in Hepa1c1c7 cells. DOPAC not only induced the gene expression of ALDHs, but also the phase 2 genes including glutathione S-transferase, heme oxygenase-1 and glutamate-cysteine ligase, catalytic subunit, both of which play an effective role in protection against oxidative stress (Ghattas et al., 2002)(Nakamura et al., 2000). Oxidative stress is also suggested to be involved in the toxic mechanism of acetaldehyde (Matysiak-Budnik et al., 1996). Therefore, the DOPAC-induced phase 2 enzymes cannot be ruled out in the cytoprotective mechanism against acetaldehyde. Acetaldehyde can impair the protein functions and gene expression by covalent binding formation with proteins and DNA (Ishii et al., 2009). Glutathione is also a binding target for acetaldehyde and inhibits its electrophilic reactivity (Setshedi et al., 2010). However, the treatment of Hapa1c1c7 cells with lower than 100 μM of DOPAC did not exhibit a significant alteration of the intracellular level of reduced form of glutathione (Tang et al., 2016). Therefore, the glutathione-dependent mechanism might be unattributable to the DOPAC-induced cytoprotection against acetaldehyde.

In conclusion, we identified a major catabolite of quercetin glycosides, DOPAC, as an ALDH activity enhancer. The DOPAC pretreatment also increases the resistance to the acetaldehyde-induced cytotoxicity, possibly through the transcriptional regulation of the ALDH genes by Nrf2 and AhR. Since DOPAC is a phenolic acid catabolite of dopamine as well as quercetin with a much lower cytotoxicity (Fig. 3.5A), DOPAC has some advantages for application as a food chemical to prevent humans from an alcohol-induced abnormal reaction. Future efforts will be concerned with further understanding the signaling pathway of the ALDH induction as well as in vivo significance of the protective effect of DOPAC against the alcohol-induced toxicity.

CONCLUSION

In the present study, the mechanism of BITC and DOPAC induced ALDH activity were investigated. The cell lines we used are mouse hepatoma Hepa1c1c7 cells, human hepatocellular carcinoma HepG2 cells and mouse live normal RL34 cells.

In Chapter 2, the ability and mechanism of benzyl isothiocyanate (BITC) enhanced ALDH activity were examined.

(1) BITC incubation for 24 h, 24 h and 3 h dose-dependently increased total ALDH activity in Hepa1c1c7 cells, RL34 cells and HepaG2 cells.

(2) BITC increased both gene and protein expression of ALDHs.

(3) BITC increased Nrf2 expression and stimulated its nuclear translocation.

(4) Silencing Nrf2 decreased total ALDH activity and BITC-induced gene expression of ALDH2 and ALDH3A1.

(5) BITC mitigated acetaldehyde-induced cytotoxicity through Nrf2 pathway and partly through the ALDH3A1 up-regulation.

In Chapter 3, I investigated DOPAC induced ALDH activity in Hepa1c1c7 cells.

(1) DOPAC up-regulated total ALDH activity.

(2) DOPAC not only up-regulated gene expression of ALDHs, but also upregulated protein expression of ALDHs.

(3) The DOPAC pretreatment also increases the resistance to the acetaldehyde-induced cytotoxicity, possibly through the transcriptional regulation of the ALDH genes by Nrf2 and AhR.

Taken together, these series of studied suggested the induction of ALDH activity of BITC and DOPAC can mitigated acetaldehyde-induced cytotoxicity through some transcriptional pathway such as Nrf2, AhR or NF-κB.

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